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Developing and testing of an air dilution flow olfactometer with known rates of concentration change

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Abstract

Background—Concentration is a variable aspect of an odor signal and determines the operation range of olfactory receptor neurons (ORNs). A concentration increase is perceived as an odor stimulus. The role that the rate of concentration increase plays thereby has been studied with electrophysiological techniques in ORNs of the cockroach. A key prerequisite for these studies was the development of an air dilution flow olfactometer that allowed testing the same change in concentration at various rates.

New method—The rate of concentration change was controlled and varied by changing the mixing ratio of odor-saturated and clean air by means of proportional valves. Their input voltages were phase shifted by 180° to hold the mixed air at a particular constant volume flow rate.

Results—Using this stimulation technique, we identified, in a morphologically distinct sensillum on the cockroach's antenna, antagonistically responding ON and OFF ORNs which display a high sensitivity for slow changes in odor concentration.

Comparison with existing methods—The olfactometer is unique because it enables delivering slowly oscillating concentration changes. By varying the oscillation period, the individual effects of the instantaneous odor concentration and its rate of change on the ORNs' responses can be determined.

Conclusions—The olfactometer provides a new experimental approach in the study of odor coding and opens the door for improved comparative studies on olfactory systems. It would be

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Ethics statement

All the experiments described in the manuscript were performed with laboratory-reared insects. No special permit was required. After experiments, cockroaches were quickly killed by freezing. All institutional and national guidelines for the care and use of laboratory animals were followed.

Declaration of conflicts of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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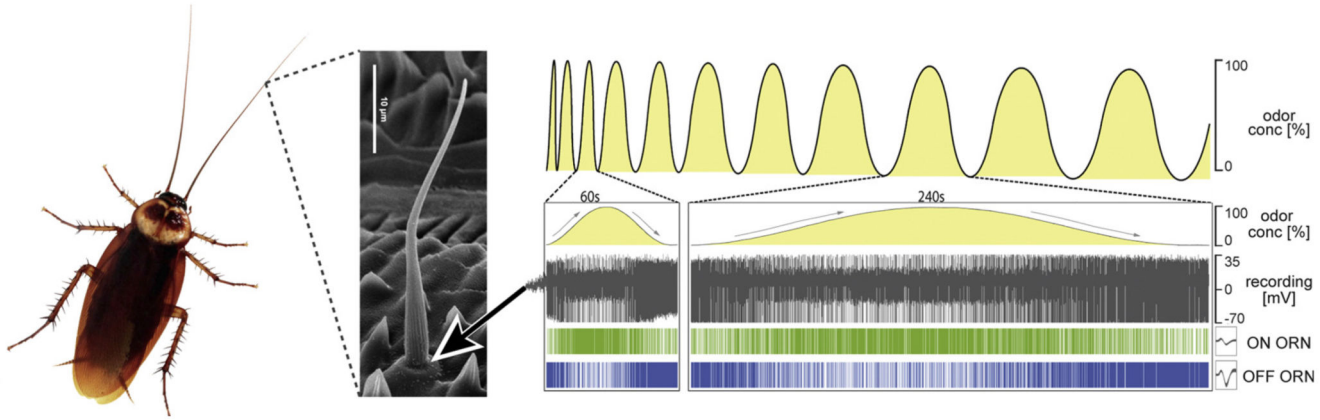
important to gain insight into the ORNs' ability to detect the rate of concentration change in other insects that use odors for orientation in different contexts.

Abstract



Graphical abstract.

Keywords

Electrophysiology; Insect; Odor coding; Proportional valves; Feedback controller; miniPID

1 Introduction

The significance and implications of experimental findings to understand how olfactory systems help animals locating odor sources is strongly dependent on the nature of the stimulus delivery system and the accuracy with which it can be controlled. Therefore, the mode of stimulus delivery to an olfactory sense organ has to be selected according to the objectives of the research. Electrophysiological recordings from insect olfactory receptor neurons (ORNs) were largely aimed at determining mechanisms of encoding odor identity and concentration or to describe at least what odor information is contained in the ORNs activity. While there is increasing evidence that the code of odor identity is consistent with an "across-fiber" odor coding strategy first suggested by Pfaffmann (1959) and Erickson (1963), quantitative data on encoding fluctuating concentration changes are scarce.

In a turbulent odor plume, an insect encounters the dispersing chemical signal as concentration pulses of varying shapes and heights interspersed with periods of low or zero concentration (Murlis and Jones, 1981; Moore and Atema, 1991; Zimmer-Faust et al., 1988, 1995). This concentration pattern creates an "odor landscape". The main features are that closer to the source, the mean pulse amplitude and the correlated steepness of the pulse slope increase while the repetition rate and the duration of inter-pulse periods decrease (Atema, 1996). Plume-tracking lobsters use the instantaneous distribution of the pulse amplitude and the slope steepness, or equivalently the rate at which pulse concentration increases, as distance and directional information for finding the source (Atema, 1985, 1995, 1996; Moore and Atema, 1991). Electrophysiological studies have

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shown that chemoreceptors in the aesthetasc sensilla on the lobster´s lateral antennular filaments function as “concentration slope” detectors which respond differently to a range of pulse onset slopes or rates at which concentration increases (Devine and Atema, 1982). From the perspective of a marine crustacean, the problem of tracking turbulent odor plumes is thus a temporal one, requiring detecting and quantifying the steepness or the rise time of the encountered odor pulses for guidance to their source.

In studies on insect olfaction, variations in the pulse onset slope have not received the same attention as variations in the peak concentration, the duration and the repetition rate of odor pulses. The rationale was that the intermittent nature of the turbulent odor plume, in particular the frequency of encountered odor pulses, is important for mediating plume tracking and sustained upwind flight in males of several moth species (Mafra-Neto and Cardé, 1994, 1996; Murlis, 1997; Zimmer-Faust et al., 1988, 1995; Murlis and Jones, 1981; Webster and Weissburg, 2001; Moore and Crimaldi, 2004; Koehl, 2006; Vetter et al., 2006; Vickers, 2006; Riffell et al., 2008). To simulate wind-borne odor filaments, odor stimuli were provided as brief on-off (square) pulses. The time course of pulse concentration was not considered as a stimulus parameter. However, square pulses with constant rise time produce different rates of concentration increase if the amplitude is different: the higher the concentration amplitude, the faster the concentration increases during the same transient time. Thus, the higher discharge to a pulse of higher concentration may be due to the increase in concentration or to the increase in the rate of change. The use of step-like concentration changes adds another potential parameter, namely the background concentration level from which the step was initiated (Borroni and Atema, 1988; Kelling et al., 2002; Kim et al., 2011; Burgstaller and Tichy, 2011; Hellwig and Tichy, 2016). Because it is extremely difficult to measure the rate of concentration increase at the sensillum upon the arrival of a rectangular, transient concentration front, the odor stimulus was usually defined by the peak or mean concentration of the pulse or by the difference between the concentration level from which the step was initiated and the concentration of the pulse.

Are transient concentration changes adequate for describing the performance of an olfactory system? If the rate of concentration increase is so great that the ORN activity is no longer influenced by reducing the duration of a concentration increase (i.e., the onset slope of the concentration pulse), but only by raising the amplitude of the concentration increase, then pulse concentration is adequate for describing the odor stimulus. In the case of the cockroach ON and OFF ORNs, however, pulse concentration is definitely inadequate as a stimulus parameter. This can be shown by keeping the amplitude of the concentration change constant and extending the concentration rise time at the sensillum to seconds instead of milliseconds. Slowing down the concentration rate lowers the discharge rate even when the concentration amplitude remains constant (Tichy et al., 2005; Hellwig and Tichy, 2016; Tichy and Hellwig, 2018).

To control and vary the rate of concentration change, we invented a dilution flow olfactometer that delivered not only abrupt, pulse-like concentration changes but also slow and continuous ramp-like or oscillating concentration changes with different durations. By testing slow and continuous up and down concentration changes, we identified in a morphologically distinct sensillum on the cockroach’s antenna two types of ORNs which

display antagonistic ON and OFF responses. Increasing odor concentration raises impulse frequency in the ON ORN and lowers it in the OFF ORN. Correspondingly, contrary effects are produced by decreasing odor concentration (Hinterwirth et al., 2004; Tichy et al., 2005; Burgstaller and Tichy, 2011, 2012; Hellwig and Tichy, 2016; Tichy and Hellwig, 2018). The odor of lemon oil was chosen as food odor stimulus because the response spectra of ORNs and antennal lobe neurons were examined using this odor (Boeckh, 1974; Sass, 1978; Selzer, 1981, 1984; Zeiner and Tichy, 2000), and the components of the odor mixture were quantitatively identified (Günther, 1968; Shaw, 1979), facilitating a comparison with these studies. The same antagonistic responses were obtained by odors released from various ripe fruits like lemon, orange, apple and banana. Baked bread elicited weak antagonistic responses, but meat and cheese no response. The ON and OFF ORNs are highly sensitive to two independent components of the fruit odor stimulus: the instantaneous odor concentration and its rate of change. In both ORNs, gain control acts as a trade-off between the sensitivities to instantaneous concentration and the rate of change. When the concentration oscillates rapidly with brief periods, adaptation improves the gain for instantaneous odor concentration and reduces gain for its rate of change. Conversely, when the concentration oscillates slowly with long periods, adaptation increases gain for the rate of change at the expense of the instantaneous concentration.

ON and OFF ORNs which produce excitatory responses to concentration increments and decrements are so far unique to the cockroach. It is hard to imagine that they do not exist in other insects as well. However, the identification of ON and OFF ORNs seems to depend on the stimulation technique. In previous electrophysiological studies, odor stimulation consisted of brief concentration pulses separated by clean-air intervals. This form of stimulation was especially suitable for eliciting excitatory responses in ON ORNs, in particular when the duration of the stimulus-free, clean-air interval was long enough to allow adequate recovery and conditioning of the ON ORNs on the next brief on-pulse. On the other hand, the brief on-pulses, which are the stimulus-free, odorized-air interval for the OFF responses, may not allow adequately recovery and conditioning the OFF ORNs on the following clean air stimulus. Of course, an adequate conditioning interval prior to the odor OFF stimulus is necessary for eliciting a significant OFF responses. We solved the problem of adequate conditioning of both ORNs by developing a novel air dilution flow olfactometer. This instrument allowed presenting not only brief and abrupt concentration changes but also slow and continuous concentration changes, and in addition, maintaining concentration at any level for arbitrary durations.

Our novel air dilution flow olfactometer uses two computer-controlled electronic proportional valves, each in series with a volume flow meter, and a *PI* (Proportional–Integral) controller for linearizing the output air flow rate of each valve. A Photo-Ionization Detector (miniPID, Aurora Scientific) inserted into the path of the output demonstrates close consistency of the instantaneous concentration of the stimulating air stream with the values desired by setting the two valves. Here we describe technical details of the device and provide the wiring diagram for the *PI* controller specific enough to be used as a template. We validate the performance of the dilution flow olfactometer using electronic flow meters and a photoionization detector, and assess their consistency with the recorded discharge rates of the ON and OFF ORNs. We present new electrophysiological data of the ORNs

concerning the effect of high and low amplitude oscillating concentration changes performed from different background concentration levels. These experiments mimic concentration distribution inside turbulent odor plumes or at the plume boundary region, which is characterized by concentration patterns with regions of high concentration interspersed by periods of low or zero concentration. We hope this stimulates similar studies on other insects in order to learn whether different adaptations exist to different contexts of odor-guided orientation behavior.

2 Material and methods

2.1 The experimental animal and the food odor selected for studying the ON and OFF ORNs

The cockroach Periplaneta americana is a peridomestic species with an acute chemical sense, often found in storage rooms and garbage sites. The peripheral olfactory system has been extensively studied (reviews: Boeckh and Ernst, 1987; Boeckh et al., 1990), resulting in a fairly complete list of olfactory sensilla, their structures, innervation patterns and distributions on the antennae (Sass, 1972, 1976, 1978; Altner et al., 1977, 1983; Toh, 1977; Schaller, 1978; Fujimura et al., 1991). The response spectra of many ORNs to natural food odors and to a selected repertoire of chemically pure substances have been explored in considerable depth based on electrophysiological recordings.

The odor of lemon fruit is highly effective in activating ORNs as well as antennal lobe neurons (Boeckh, 1974; Sass, 1978; Selzer, 1981, 1984). It contains many different compounds belonging to several chemical classes (Günther, 1968; Shaw, 1979). Because the sensory quality of the compounds in natural fruits can differ considerably depending on the region of origin and source of import, maturity and storage, synthetic lemon oil (Roth, D ~ 0.85, Art. 5213.1) has been used as a standardized fruit odor stimulus for ORNs (Sass, 1978; Selzer, 1981, 1984; Zeiner and Tichy, 2000; Hinterwirth et al., 2004; Tichy et al., 2005; Burgstaller and Tichy, 2011, 2012) as well as for local neurons and projection neurons in the antennal lobe (Boeckh, 1974; Zeiner and Tichy, 2000).

2.2 Morphological structure and sensillum location

The long antenna of the cockroach Periplaneta americana consists of 120–180 ring-shaped segments which grow thinner and longer with increasing distance from the head. The olfactory sensilla are sufficiently apart to allow electrophysiological recording from individual, morphologically distinguishable types without interference from neighboring sensilla. This has helped sensillum classification based upon sensilla characteristics such as wall structures, presence and location of pores, and numbers of ORNs. Schaller (1978) distinguished three types of single-walled sensilla (swA, swB, and swC) and two types of doublewalled sensilla (dwA and dwB). The swA and swB sensilla were also assigned to the basiconic sensilla and the swC sensillum to the trichoid sensilla (Toh, 1977). The swA and swC sensilla are innervated by two ORNs; the swB sensillum by four. The ORNs in the swA sensilla and in about halve of the swB sensilla respond to lemon odor, the ORNs in the remaining swB sensilla are activated by the female sex pheromone periplanone (Schaller, 1978). The trichoid swC sensilla contain a pair of ON and OFF ORNs, which

provide excitatory responses to an increase and decrease in the concentration of lemon odor, respectively (Burgstaller and Tichy, 2012; Tichy and Hellwig, 2018). In studying the effect of the rate of concentration change on the ON-ORN's discharge, the always pressing and nearly always elusive question was the effect of the background concentration on the response to slow rates of change. Key features for a walking plume tracker locating an odor source are the profile of the pulse slopes as well as the level of the background concentration between the odor pulses which increases with decreasing distance to the source. The present investigation is focused on the simultaneous dependence of the ON and OFF ORNs on the background level and their superimposed concentration fluctuations. All recordings were taken from the swC sensillum, which is about 3 μm in diameter and 30−40 μm in length (Fig. 1).

2.3 Preparation and electrophysiological recording

The adult male cockroaches were slightly anesthetized with CO_2, positioned dorsal-side-down on a tightly fitting Perspex holder with the head and the antennae protruding. The head was fixed in this position with a notched Perspex yoke placed between the head and the thorax. Wings and legs were immobilized with strips of Parafilm wrapped around the holder. One antenna was restrained with dental cement (Harvard Cement) to the edge of a narrow Perspex ridge extending from the holder. Then the holder was placed in the experimental set-up so that the odor delivery nozzle was at a distance of 10 mm from the recording site in the middle of the antenna. Electrolytically sharpened tungsten metal electrodes were used for recording action potentials. The reference electrode was inserted longitudinally into the tip of the antenna. The recording electrode was forced into the base of the sensillum just deeply enough to establish electrical contact with the ORNs. The signals from the electrodes were amplified, band-pass-filtered (0.1−3 kHz), passed through a CED1401-micro (Cambridge Electronic Design, 12 bit, 300 kHz) interface connected to a PC for on-line recording. The data were stored on a hard disc and analyzed off-line using commercial software (Spike2).

The spikes were detected using a standard template matching procedure. Each spike was compared against the templates, and each time a template was confirmed it was added to templates by overdrawing. Adding a spike to a template may change the shape and width of the template outlines. Thus, the template boundaries displayed the degree of homogeneity of spike classification and informed about temporal peculiarities of the spike trains, such as a gradual change from one class to the other, resulting in erroneous counts in each class. Impulse frequency (imp/s) was calculated from running averages of three consecutive 0.2-s periods (Burgstaller and Tichy, 2012).

2.4 Dilution flow olfactometer

The dilution flow olfactometer described here offers some unique features concerning stimulus control and delivery. These include: *1*) continuous flow of the odor-loaded air for temporal control, *2*) flexible selection of the background concentration level and the rate at which concentration increases and decreases, *3*) ease of operation by computer-controlled proportional valves, and *4*) a *PI* controller to linearize the output air flow rate of each valve. A schematic diagram of the complete system is shown in Fig. 2.

2.4.1 Design characteristics—A stream of compressed, pre-cleaned and pre-dried air from the laboratory line was passed through an adsorption drier (DPS 1–8A; Filtrations-Separations-Technik, Essen, Germany). The stream was then split into two equal-sized channels. Manual needle valves in series with calibrated flow meters of the Rotameter type controlled their flow rates. Stream A was odorant-loaded by bubbling through many tiny holes in polyethylene tubing fixed at the bottom of a 25-l tank containing 100 ml of the undiluted liquid odor of lemon oil. Stream B was conducted through an empty tank of the same design and remained clean. After leaving the tanks, each air stream was passed through an electrical proportional valve (Rotary Slide Servo Valve; Kolvenbach KG, KWS 3/ 4) in series with an electronic flow meter calibrated by the manufacturer for volume flow rate (AWM 3000, Honeywell). The slowly oscillating concentration changes were produced by a 180° phase shift of the valves' control voltages (D-A converter, 1401plus, Cambridge Electronic Design). Thus, the total volume flow rate of both air streams was held constant at 1.5 m/s as the underlying odor/clean-air ratio was varied in a sinusoidal manner. This ratio was regulated by the output sequencer function of the data acquisition software (Spike2, v.3.18; Cambridge Electronic Design [CED], Cambridge, UK), using a self-written sequencer script. A feedback controller compared the signals from the flow meters with the set point voltages to the proportional valves and counteracted any deviations of the flow rate set by the output sequencer. If both valves are closed irregularly at the same time, a safety valve protects against overpressure. For stimulation, the mixed air stream emerged from a 7-mm-diameter nozzle at a distance of 10 mm from the recording site. The air around the antenna was continually removed by a suction tube at a speed of 2 m/s.

2.4.2 Feedback controller—A feedback controller was designed to set the actual flow rate of air through each proportional valve at the desired flow rate specified by the output sequencer of the CED. The actual output flow rate can deviate from the desired setpoint flow rate (reference input, $r(t)$) as a result of the valve's non-linear flow characteristics at the low pressure air flow used here, as well as due to the tubing system or the volume flow rate used to regulate the air flow velocity. A potential deviation or error ($e(t)$) was detected by determining the difference between the measured output flow rate ($y_m(t)$) and the setpoint flow rate ($r(t)$). The controller converted the error into a control action ($u(t)$) to force the output flow rate back towards the desired setpoint flow rate.

We implemented a PI (proportional-integral) controller. The block diagram of the air flow rate under PI control and the control algorithm of the output signal ($u(t)$) are shown in Fig. 3. By adjusting the gain settings of the proportional and integral controllers (Kp and Ki, respectively), the effect of the controller on the output can be varied. The proportional gain (Kp) amplified the waveform output voltage of the CED according to the required input voltage of the proportional valve. Furthermore, higher Kp values tended to cause the system to react more quickly and to respond more rapidly to errors away from the setpoint. The integral gain (K_i) of the controller reduced steady errors by increasing the control signal and driving the error down. The circuit diagram is shown in Fig. 4.

The digitized output voltages of the electronic flow meters were used to display the time course of the flow rates of the odor-loaded and clean air streams, and also of the mixed air stream representing the temporal profile of the odor concentration during stimulation. Odor

concentration was calculated from the flow rate ratio of the two air streams and indicated by the percentage of odor-loaded air in the stimulus air stream. “0 % concentration” means clean air only and indicates that the air stream directed onto the antenna is not mixed with the odor-loaded air, while “100 % concentration” means pure odorized air (stimulus air not mixed with clean air).

2.4.3 Olfactometer performance—A photoionization detector (200A miniPID, Aurora Scientific, bandwidth 330 Hz) was used to verify that mixing of the odor-loaded with the clean air stream produces the required slowly oscillating concentration changes. The flow meters were located directly downstream of the proportional valves and controlled both timing and amplitude of the phase-shifted oscillating air streams within the delivery tubes (Fig. 2). The miniPID needle was adjusted in the center of the mixed air stream, midway between the output nozzle and the antenna (Fig. 2). The miniPID signal provides high-frequency measurements of the compound’s concentration drawn through the detection cell. As the miniPID was not calibrated with a reference gas at known absolute concentration, the output voltage of the miniPID indicated the relative odor concentration. Nevertheless, high-frequency measurements of the air stream’s relative concentration at a distance of 5 mm to the antenna, or 3.3 ms ahead of it, were extremely valuable for testing the performance of our olfactometer.

Two characteristics of the olfactometer were determined: (*1*) the balance between air pressure and flow velocity, and (*2*) the ability of the delivering system to repeatedly generate concentration changes at different background concentration levels in addition to the moment-to-moment stability of the output concentration.

(1) Air pressure was used to regulate the flow velocity of the stimulating air stream. The higher the air pressure, the faster is the flow velocity and the less is the effect of mixing the homogenous air stream emerging from the nozzle with the surrounding still air. High air pressure will build up at totally or partly shut off proportional values. Indeed, when the valves were opened rapidly in a step-like manner and then kept at a constant level, the flow rates through the valves rapidly increased to a brief peak, followed by a decrease to a constant flow level which corresponds to the valve’s throughput conditions (Fig. 5). Peaking of the air flow rate immediately after a rapid opening and closing of the valves is measured and monitored with both the flowmeters (Fig. 5Aa, b) and the miniPID (Fig. 5Ae). The air flow peaks produced transient overshoot in the mixed air stream and in the odor concentration (Fig. 5Ac, d). Here, the concentration peaks were too fast to be compensated by the *PI* controller. By contrast, when the valves were activated in a sinusoidal manner, the flow rates and the resulting instantaneous concentration did not peak, as indicated by the smoothly oscillating signals of the flowmeters and of the mini PID (Fig. 5B). The vertical shifts of the flowmeter and miniPID signals result from the change of the concentration range at which the oscillations were performed. In the present study, we used sinusoidal concentration changes.

(2) The reproducibility of the flow rate of the mixed air stream and at a given pressure was tested between several experiments on one day or successive days. Typical examples of a series of constant-amplitude, oscillating concentration changes with different oscillation periods (6 s, 30 s, and 120 s) and at different background concentrations are given in Fig. 5B. Visual inspection of the time course of odor concentration obtained by the electronic flowmeters revealed that repeated oscillations with the same period closely match in timing even when covering different concentration ranges (Fig. 5Ba, b).

While the timing of the maxima and minima values of the oscillating signals of the electronic flowmeter and also the thereof calculated concentration match with those of the oscillating absolute miniPID signal, the amplitude of oscillating miniPID signal tended to become smaller with increasing concentration range (Fig. 5Bd). Thus, in the high concentration range, the effect of oscillating concentration change on ORN response will be underestimated. No systematic attempt was made to determine whether the divergence between the oscillating PID signal and the oscillating flow-meter signal affects the oscillating activity of the ORNs. We periodically cleaned the lamp window inside the PID cell to remove contamination which absorbs some of the energy, but the sensitivity of the output signal of the PID system also decreased over time. Therefore, the absolute miniPID values were normalized such that the maximum value obtained in an experiment was arbitrarily set to the value “1”.

3 Theory

Praha et al. (1968) stated in their comprehensive review of Air Dilution Olfactometers that “*When there is a need for a high degree of control over odorant concentration and, possibly, the total quantity of odorant molecules or the temporal parameters of the stimuli entering the nose, air dilution olfactometry is needed. In this approach, a continuous odorant concentration is controlled and varied, typically, by changing the relative volume flow rates of clean and odor–saturated air.” The system’s performance is evaluated by “real–time measurements of the arrival of the odorant at the subject’s odor or as a function of odorant identity and concentration*.” Praha et al. (1968) also noted that “*this can be done by using a photoionization detector (PID)*.” Notwithstanding the encouraging presentation of various types of dilution flow olfactometers, the rate of concentration change was recognized as a parameter of the odor stimulus, but its effect on smelling the odor was not quantified. In more recent electrophysiological studies, a photoionization detector was routinely employed to monitor and measure how the rise time of the odor stimulus varies with the odor identity, the flow rate and the concentration. However, no attempts were made to analyze the effect of the rate of concentration change on the responses of ORNs or higher order olfactory neurons. Accordingly, neural activity has never been plotted as a function of the rate of concentration change, and this function has not been quantified. The reason seems to be that the air flow rate, air pressure and tubing produce the effect that a linear voltage change (used to control the action of the proportional valves) results in a non-linear output flow rate. We therefore developed a *PI* feedback controller that linearizes valve action. The novel air

dilution flow olfactometer generates not only known and verifiable odor concentrations at different constant flow rates but also known and verifiable rates of concentration changes.

4 Results

Slowly oscillating concentration changes have been presented at zero background concentration in order to determine the effect of the rate of concentration change on the ORNs responses changes in the concentration of lemon oil odor (Burgstaller and Tichy, 2012). The single-sensillum recording in Fig. 6 shows that the rate of discharge of the ON ORN is increased by raising odor concentration and decreased by reducing it. Conversely, the discharge rate of the OFF ORN is increased by reducing odor concentration and decreased by raising it. Moreover, the frequency maximum of the ON response leads the concentration maximum, and the frequency minimum the concentration minimum; conversely, the frequency minimum of the OFF responses leads the concentration maximum, and the frequency maximum the concentration minimum. Because of this phase shift, the oscillations in odor concentration cannot fully explain the oscillations in impulse frequency. A second stimulus component that is in advance of the oscillating concentration change must affect the responses. The rate of change, as the first derivative of concentration, is the obvious parameter. For the sake of argumentation, some other parameters might also influence the responses (beyond the instantaneous concentration and its rate of change). Potential candidates include the level of the background concentration from which the oscillations are initiated and the amplitude of the concentration oscillation.

The four parameters of the odor stimulus – the instantaneous odor concentration and its rate of change (which can be varied independently by changing the oscillation period), the background concentration level and the oscillation amplitude – can be combined in different ways. Nonetheless, to keep the duration of the experiments on individual pairs of ON and OFF ORNs within reasonable limits without reducing the significance of the results or their validity, we used a 4-step protocol exemplified in Fig. 7. It starts with a large-amplitude oscillation of 60 % at a zero background concentration, followed by two small-amplitude oscillations of 25 % initiated first at 0% and then at 25 % background concentrations, and finally a 20 % oscillation presented at a 45 % background concentration.

Fig. 7A, B shows the time course of both the oscillating concentration change and the oscillating rate at which concentration changes in the 4-step protocol. Note that the rate of change achieves the highest value of ± 5%/s during the large-amplitude oscillations but only ± 2.5 % during the small-amplitude oscillations. Impulse frequency of the ON ORN rises to 10 imp/s, sometimes higher, and the OFF responses reach values of 20–25 imp/s (Fig. 7C, D). In addition, the frequency maxima and minima are more pronounced in the OFF versus ON ORN, and the frequency minima achieve lower levels in the ON ORN. While at background concentrations of 20 % and 45 % the frequency maxima and minima tend to be smoothed in the ON ORN, they are clearly developed in the OFF ORN. The smoothed ON ORN responses to oscillating concentration changes at 20 % and 40 % background occur at slightly higher frequencies than the pronounced responses at 0% background. In the OFF ORN, the frequency minima of the oscillating responses to small amplitude concentration changes are slightly higher than to large amplitude changes, with no obvious influence of the

background concentration. However, the response maxima to small-amplitude oscillations are lower at background concentration of 20 % and 45 % than at 0% background (Fig. 7D).

Formally, the lack of pronounced response maxima and minima of the two ORNs during small-amplitude concentration oscillations could depend on the high background concentrations or on the low rates of change. Varying the rate of concentration change by varying the duration of the oscillation period may help differentiate between the effects of the two parameters. To this end, the 4-step protocol was tested with oscillation periods of 6 s, 30 s and 120 s. Figs. 8 and 9 are examples of the response of a single ON and a single OFF ORN shown as 3-D curves generated by plotting impulse frequency as a function of the instantaneous odor concentration and its rate of change. The closed curves correspond to Lissajous figures, which occur when two sine waves of equal frequency are combined. Their shapes depend on the phase between them and their relative amplitudes. Multiple regressions ($F = y_0 + a\,dC/dt + b\,C$; where F is the impulse frequency and y_0 the height of the regression plane) were calculated to determine the simultaneous effect of instantaneous concentration (b-slope) and the rate of change (a-slope) on the impulse frequency for all possible combinations of the different parameters.

The slopes of the multiple regressions demonstrate the two properties that characterize the ON and OFF ORNs: (*1*) the sign of the slopes is positive for the ON ORN and negative for the OFF ORN; that is, an increase in both instantaneous concentration and its rate of change raises the impulse frequency of the ON ORN and lowers that of the OFF ORN; (*2*) the slopes are steeper for the OFF than for the ON ORN; that is, given changes in instantaneous concentration or in the rate of concentration change have stronger effects on the frequency of the OFF than on the ON ORN with due consideration of the sign. Poor R^2 values reflect the small frequency ranges close to the bottom of the frequency spectrum available for the oscillating discharge rates at small-amplitude oscillations. The frequency range of both ORNs becomes even further narrowed when the background concentration level is raised. Nevertheless, a double dependence can be obtained in the ON ORN for large-amplitude oscillations presented in combination with the 6-s oscillation period (Fig. 8Aa), and in the OFF ORN for both large- and small-amplitude oscillations at different background concentrations in combination with the 6-s oscillation period (Fig. 9Aa–Ad).

The slopes of the multiple regressions indicate the gain of responses of an ORN for the instantaneous concentration and its rate of change. In the ON ORN example, shown in Fig. 8Aa, for a large-amplitude oscillation with a period of 6 s, the gain for the instantaneous concentration is 0.14 imp/s per 1%, and the gain for the rate of concentration change is 0.04 imp/s per 1%/s. Thus, during a 6-s oscillation period, impulse frequency of the ON ORN can be influenced more by changing the instantaneous concentration by an additional percent than by changing the rate of change by 1 %/s. In the OFF ORN example in Fig. 9Aa for a large-amplitude oscillation with a period of 6 s, the gain for the instantaneous concentration is −0.51 imp/s per 1 %, and the gain for the rate of change is −0.31 imp/s per 1%/s (the negative values represent the downward direction of the concentration change, yielding a rise in impulse frequency of the OFF ORN). The gain for the instantaneous concentration is -0.80 imp/s per 1% and the gain for the rate of change is -0.60 imp/s per 1%/s. Thus both gain values of the OFF ORN are slightly increased when the

concentration amplitude at 0% background concentration is decreased. However, the gain values for both parameters are reduced when small-amplitude oscillations are presented at the high background concentration of 45 % (Fig. 8Ad). In this situation, the gain for the instantaneous concentration is -0.47 imp/s per 1% and the gain for the rate of change is -0.36 imp/s per 1%/s. Hence, the impulse frequency of the OFF ORN can be influenced more by changing the instantaneous concentration by an additional percent than be changing the rate of concentration change by 1 %/s.

We determined the dependences of the gain for the instantaneous concentration and its rate of change on the combined effects of the oscillation amplitude, the background concentrations and the oscillation period by a one-way ANOVA test for pooled regression planes of 6 ON and 6 OFF ORNs. The entries in the matrix in Fig. 10A, B are the mean gain values and SD obtained for all possible combinations of the three oscillation periods (*rows*, I-III) with the three concentration amplitudes at the three background concentrations (*columns*, *a-h*). Significant differences between two gain values at a p-value better than 0.05 are indicated in Fig. 10C, D by double arrows: (i) horizontally directed between the gain values at different amplitudes and background but presented at the same oscillation period, and (ii) vertically directed between the gain values at different oscillation periods but presented at the same amplitude and the same background.

In the ON ORN, the horizontal double arrows show differences in the gain values for the rate of concentration change between different oscillation amplitudes depending on the background concentration and the oscillation period: for small amplitudes presented at 0 % and 20 % as well at 0% and 45 % backgrounds, provided that the period was 30 s (Fig. 10AIIa-d and CIIa-d); and also for the large and the small amplitude at 0% and 45 % backgrounds, with periods of 30 s and 120 s, respectively (Fig. 10AIIa, d, IIIa, d and CIIa, d, IIIa, d). Different gain values for the instantaneous concentration were obtained between small-amplitude oscillations at 0% and 45 % backgrounds when the oscillation period lasts 6 s (Fig. 10AIf, h and CIf, h).

In the OFF ORN, the horizontal double arrows indicate different gain values for the rate of concentration change between small-amplitude oscillations when tested at 0% and 20 % as well at 0% and 45 % backgrounds with 6-s periods (Fig. 10BIb-d and DIb–d). Different gain values for the instantaneous concentration exist between large- and small-amplitude oscillations when presented at 0% background, and between small-amplitude oscillations at 0% and 20 % as well at 0% and 45 % backgrounds − all with 6-s oscillation periods (Fig. 10BIe-h and DIe-h).

In the ON ORN, the vertical double arrows display differences in the gain values for the rate of concentration change in the following cases: between oscillation periods of 6 s, 30 and 120 s, when the oscillation amplitude was large and tested at 0% background (Fig. 10AI-IIIa and CIV-VIa), and between periods of 6 s and 30 s as well 6 s and 120 s, when the amplitude was small and the background 0% (Fig. 10AI-IIIb and CIV-VIb). Different gain values for the instantaneous concentration were obtained between the three different oscillation periods when large oscillations were presented at 0%background (Fig. 10AI-IIIe and CIV-VIe), between periods of 30 s and 120 s as well 6 s and 120 s, provided that the

amplitude was small and the background 0% (Fig. 10AI-IIIf and CIV-VIf), between periods of 6 s and 30 s as well 6 s and 120 s, when a small amplitude was tested at a background 20 % (Fig. 10AI-IIIg and CIV-VIg), and between periods of 30 s and 120 s, when a small amplitude was coupled with the 45 % background (Fig. 10AII-IIIh and CV-VIh).

In the OFF ORN, the vertical double arrows indicate different gain values for the rate of concentration change between oscillation periods of 30 s and 120 s as well 6 s and 120 s, when large oscillation amplitudes were tested at 0% background (Fig. 10BIV-VIa and DIV-VIa), and between periods of 6 and 120 s, when the amplitude was small and at 0% background (Fig. 10BIV-VIb and DIV-VIb). Different gain values for the rate of concentration change were also obtained for periods of 6 s and 30 s as well 6 s and 120 s, when the oscillation amplitude was small and presented at 20 % background (Fig. 10BIV-VIc and DIV-VIc), and between 30 s and 120 s as well 6 s and 120 s, when the amplitude was large and at 45 % background (Fig. 10BIV-VId and DIV-VId,g). In the OFF ORN, different gain values for the instantaneous concentration were obtained between the different oscillation periods, provided that the oscillations were coupled with 0% background (Fig. 10BIV-VIe and DIV-VIe), and between periods of 6 and 120 s with small amplitudes at backgrounds of 0, 20 and 45 % (Fig. 10AIV-VIf-h and DIV-VIf-h).

5 Discussion

A variety of instruments has been invented for the accurate presentation of odors and they were designed to obtain a high degree of control over odor concentration and the timing of the stimulus. Most of these devices were developed to meet the demands of particular situations or unique experimental settings, each generating significant changes to existing instruments. The great variability in construction, operation and application techniques prevents us from providing a comprehensive overview of the ongoing advancements of the olfactory stimulation techniques. Instead, we refer here to olfactometers relevant to our own work on the cockroach because of their significant improvements in controlling flow rate and concentration, employing prompt rise and fall stimulation times, and allowing variable pauses of clean air. Such test procedures focused on the consistency of the concentration profile of odor pulses at a short distance beyond the output of the device (Vetter et al., 2006), the delivery of temporally complex binary mixtures (Raiser et al., 2017), the flexible control of concentration gradients varying with time (Kim et al., 2011, 2015), and the independent control and variation of odor concentration and air flow velocity (Zhou and Wilson, 2012). These olfactometers were designed to apply odor stimuli as transient on-off pulses or step-like concentration changes. The standard notion about ORN responses was that an odor elicits a level of excitation in accordance with the peak or the mean concentration of the odor pulse. The rate of concentration change was not measured and the effect of this rate on the ORN response to concentration change was not determined simply by plotting impulse frequency as a function of rate. Transient concentration changes are effective stimuli because, by rapidly passing the excitation threshold, the discharge rate far outweighs the neural noise. In contrast, slowly fluctuating or creeping concentration changes hamper determining whether the neural activity results from noise alone or from a response plus noise.

The ability to detect an odor is linked to its concentration and its rate of change. This calls for precisely controlling these two mutually independent components of the stimulus with an olfactometer that can deliver at known rates. To this end we developed an air dilution flow olfactometer with the following distinguishing features: *1*) computer-controlled electronic proportional valves for mixing the odor-saturated and the clean air in any ratio and at any rate, *2*) phase shifting of the control voltages of the proportional valves by 180° to hold the mixed air stream constant at a known but variable volume flow rate, and *3*) *PI* controller to achieve linear control of the desired output odor concentration of the proportional valves.

The air dilution flow olfactometer helped identify the antagonistically responding ON and OFF ORNs on the cockroach's antenna. By delivering oscillating concentration changes with periods of different durations, we were able to instantaneously change concentration independently of the rate of change. This revealed the individual effects of the two parameters on ORN responses. Furthermore, the dilution flow olfactometer enables modulating the mixing ratio of the odor-saturated and the clean air stream at different constant air flow velocities without affecting the rate at which the mixing ratio changes (Hellwig et al., 2019). The high stability in the instrument's output flow rate almost avoids mechanical stimulation.

Our olfactometer has no lower limit for delivering slow rates of concentration change. However, rapid rates at oscillation frequencies higher than 4 Hz (periods < 0.25 s) result in a turbulent, non-smoothly mixed air stream. Furthermore, transient concentration changes cause pressure to build up at the electronic flowmeters (Fig. 5A). Nevertheless, the broad range of slow and continuous concentration changes provides great temporal flexibility and considerably increases the efficiency of the rates that can be tested. This makes the olfactometer useful for investigating the temporal relationships in olfactory processing at different levels of the olfactory system. It also offers an opportunity to study the effect of naturalistically fluctuating concentration changes at small time scales.

The main disadvantage of the novel olfactometer is that the analysis is limited to a single odor. Delivering different odors without any cross-contamination would require different sets of tanks, tubing, valves etc. This strategy quickly becomes unfeasible with an increasing number of odors, given space and cost constraints. The primary motivation for developing the olfactometer was the need to generate a broader framework for understanding odor coding by considering alternatives to the concept of brief on–off pulses. Although only cockroaches were tested, we found that the same procedure, with only minor modifications in some of the dimensions of the animal holder, can be used to establish the first derivative in the responses of insect ORNs. Our olfactometer has been extensively tested during a series of studies and is in constant use in our laboratory. The system has fulfilled its promise and continues to provide a very suitable technique for delivering natural odors, with accurate timing and quantities of stimuli delivered.

The novel air dilution flow olfactometer is capable of delivering both transient concentration changes at rates of 500 %/s (Burgstaller and Tichy, 2011) and slow and continuous upward and downward concentration changes with periods between fractions of a second to minutes at rates of ± 2%/s. At such slow rates, 30 s are needed to increase odor concentration from

0 to 100 % (Burgstaller and Tichy, 2012; Tichy et al., 2016). While the pulse stimulation technique was designed to mimic the concentration pattern present in turbulent odor plumes (Murlis and Jones, 1981; Murlis et al., 1992; Finelli et al., 1999; Vickers, 2000), the dilution flow olfactometer was developed and implemented to imitate the distribution of odor concentration within less turbulent plumes known as “odor landscapes” (Moore and Atema, 1991; Atema, 1985, 1996; Zimmer-Faust et al., 1995; Finelli et al.,1999; Moore and Crimaldi, 2004), the fluid transition of odor concentration at plume edges (Willis et al., 2008; Lockey and Willis, 2015), and the distribution of odor concentration within homogenous plumes (Geier et al., 1999) or in still air (Willis et al., 2008).

Our studies were designed to examine the ability of ORNs to detect and process slowly fluctuating changes in odor concentration with varying rates and amplitudes. The identification of the ON and OFF ORNs was possible in part because both concentration increments and decrements elicit clearly recognizable excitatory responses. Moreover, the OFF response to a given decrement is distinctly stronger than the ON response to an increment of equal strength. Despite these asymmetric responses to oscillating concentration changes, both ORNs adjust the gain of their responses for the instantaneous concentration and the rate of concentration change depending on the duration of the oscillation period. Long oscillation periods with a low rate of concentration change or gentle slope cause the gain for instantaneous concentration to decrease at the expense of the gain for the rate of change. Conversely, brief oscillation periods with a high rate of concentration change or steep slope induce the gain for the rate of change to decrease at the expense of the gain for the instantaneous concentration (Burgstaller and Tichy, 2011, 2012; Hellwig and Tichy, 2016).

In continuation of our studies on the ON and OFF ORNs, we described here the effects of the oscillation amplitude, the background concentration level and the duration of the oscillation period on the gain of responses for the instantaneous odor concentration and its rate of change. The OFF responses emerge across a wider frequency range than the ON responses, and the response profile of the former is more distinct than that of the later. The OFF ORNs produce higher frequencies to falling concentrations than the ON ORNs to rising concentrations. Therefore, both gain values of the OFF ORNs are almost double those of the ON ORNs. We confirmed this disparity for all oscillation periods, regardless of the background concentration or oscillation amplitude. In a 4-step series of 120-s oscillations, the gain of response of both ORNs for the rate of concentration change decreases with rising background concentration, though the gain for the instantaneous concentration is not affected by the background (Fig. 10ABIIIa-d, e-f and CDIII a-d, e-f). In contrast, the response gain of both ORNs for the rate of change increases when the duration of the oscillation period is extended over the rage of 6 s to 120 s. At the same time, the gain for the instantaneous concentration decreases (Fig. 10A–D). In the ON ORNs, the decrease in gain with growing period is evident during the large-amplitude oscillations at 0% background (Fig. 10AI-IIIa and CIV-VIa), and in the OFF ORNs during both large- and small-amplitude oscillations and at both low and high backgrounds (Fig. 10BI-IIIa-h and DIV-VI a-h).

The high gain values of both ORNs at large-amplitude concentration changes correspond with the broad range of frequencies in the upper part of the spectrum used for the responses.

Similarly, the low gain values at the small-amplitude oscillations are consistent with the small frequency range in the lower part of the response spectrum. Therefore, sensitivity is best at large-amplitude concentration changes at the low background range and decreases as the concentration amplitude decreases and the background rises. If sensitivity to small-amplitude changes were high, impulse frequency would change constantly, and the faster concentration changes, the faster the impulse frequency would change. For the ORNs to be of any use, a limit would seem to be essential. Without such a limit they would always signal concentration change because impulse frequency would never be steady. The advantage of this scaling is a high degree of precision for concentration changes at low backgrounds, without excessively extending the measuring scale at high backgrounds. For a cockroach that encounters a turbulent plume, the key information provided by the ORNs will be the presence or absence of the odor signal. Strong ON and OFF responses at low backgrounds will guide the cockroach's walking path while tracking the boundaries of a spreading plume in order to reach the odor source.

As the cockroach enters the plume at low background concentrations, the ORNs perceive changing concentrations with large amplitudes. The ability of both ORNs to adjust their response gain according to the duration of the oscillation period indicates that the rate at which pulse concentration is rising and falling significantly drives information transfer. Near the odor source or close to the plume's center line – where the periods of fluctuations are brief, the amplitudes small and the background concentrations high – the ON-ORNs frequency range is narrowed and the response profiles are smoothed. A high background concentration, however, may contain important information such as the proximity to the odor source. Even though fluctuations in odor concentration become less effective in exciting the ON ORNs, they elicit more pronounced responses in the OFF ORNs, which occupy a larger portion of the frequency range. Of course, fluctuations in the ON-ORNs' discharge rates indicate closeness to the odor source, even when the fluctuations are less distinct than in the OFF ORNs. When the cockroach turns to an area featuring a lower background value, the bias of the OFF responses will compensate the diminishing sensitivity of the ON ORN for concentration changes when background rises. The OFF ORN will discharge with higher rates to the same amplitude fluctuations at the same background concentration. Thus, orientation movements must be controlled by balancing between the signals of the ON and OFF ORNs. For a plume-tracking cockroach, trends in concentration change rather than exact concentration values may provide sufficient information about the distribution of the instantaneous concentration for successfully arriving at the odor source.

Much of what we have learned about how odors are encoded by ORNs stems from electrophysiological experiments in which olfactory stimuli have been delivered as brief on-off pulses. Recording the activity of ORNs while systematically varying odor identities, pulse duration and concentration has yielded an enormous amount of information about the dynamic components of the responses. Studies that used behaviorally relevant odorants provided information regarding coding schemes suitable for reconstructing stimulus identity from the evoked neural activity. Importantly, odors not only enable the selection of mates, food or sites for oviposition, but also guide animals to the odor source. Studies of insect odor-guided orientation have revealed several spatio-temporal features of the odor signal which are important for efficient plume tracking behaviors. At the same time, little attention

has been paid to the ORN's abilities to acquire information about the distribution of the instantaneous odor concentration and the local rates of concentration change. The novel air dilution flow olfactometer described here allows examining, for the first time, the ability of ORNs to detect and process slowly fluctuating changes in odor concentration at varying rates and amplitudes as well as creeping concentration change that persists in one direction of concentration change. The ability of cockroach ORNs to adjust the gain of responses for the instantaneous odorant concentration and its rate of change to the prevailing fluctuations in odorant concentration is currently unique. Future studies should investigate other insects in this regard.

Although some topics regarding the ON and OFF ORNs in the tri-choid *swC* sensilla are still outstanding, several questions remain to be solved. The process of transduction has not been investigated in the OFF ORN yet. It is not known how slowly decreasing fruit odor concentration produces an excitatory response. The ON and OFF ORNs are not a symmetrical and complementary system with equal and opposite responses. In the OFF ORN, the gain values for the instantaneous concentration and its rate of change tend to be higher than in the ON ORN (Burgstaller and Tichy, 2011, 2012; Hellwig and Tichy, 2016; Tichy et al., 2016). Decreasing concentration seems to be more important than increasing concentration in the performance of localizing food sources. The higher sensitivity of the OFF ORN could imply a lower threshold for food odors, including compounds of the complex mixtures. As the responsiveness to an odor depends on concentration, OFF ORNs with lower threshold may not only produce stronger responses to the same spectrum of compounds that stimulates the ON ORNs, but additionally weak responses to compounds that are ineffective in eliciting a response in the ON ORNs. One may expect therefore that the ON and OFF differ in their breath of tuning. Note that odor response spectra were generally tested by presenting transient concentration changes, with the advantage that when passing rapidly excitation threshold the discharge rate noticeably emerges from neural noise. With slowly fluctuating or creeping concentration changes it could be difficult to recognize the change in the discharge rate as noise or response plus noise. This possibly implies that an ORN's odor response spectrum depends on the time the rate at which odor concentration is changed. By using concentration pulses, the ORNs in the basiconic swA and swB sensilla have been assigned a role in coding odor identity, including the lemon oil (Sass, 1976, 1978; Schaller, 1978; Selzer, 1981). The question is, however, the range of concentration rates over which the lemon oil odor activates these ORNs. Gain control was not examined.

The main objective of our electrophysiological studies was to assess the temporal response characteristic of the ON and OFF ORNs and to describe quantitatively the information supplied to the brain by these ORNs regarding the rate at which fruit odor concentration changes. Such a quantitative description would not only allow a better understanding of the way in which ORNs contribute to localizing an food odor source, but would also assist in the interpretation of the neural activity recorded at higher levels along the olfactory pathway. This requires an odor delivery system with great flexibility in order to generate concentration changes at various rates in a controlled manner. Our air dilution flow olfactometer has been shown to meet these requirements.

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6 Conclusions

One of the most difficult stimulus parameters to control is the rate at which odor concentration changes, because it depends on a number of potential stimuli with which the olfactory receptor neuron is confronted. These include the mixing ratio of the volume flow rates of the clean and the odor-saturated air stream used for delivering slow and continuous concentration changes at various defined rates. Our novel air dilution flow olfactometer solves this problem by passing the two air streams through proportional valves in series with electronic flowmeters for feedback control of their relative volume flow rates. This instrument mimics the rates of concentration increase of odor pulses within a slowly expanding, uniformly distributed odor plume. Considering alternatives to the concept of transient on-off concentration pulses will help generate a broader conceptual framework for odor coding, including the impact of the rate of concentration change on the responses of olfactory receptor neurons to changing odor concentration.

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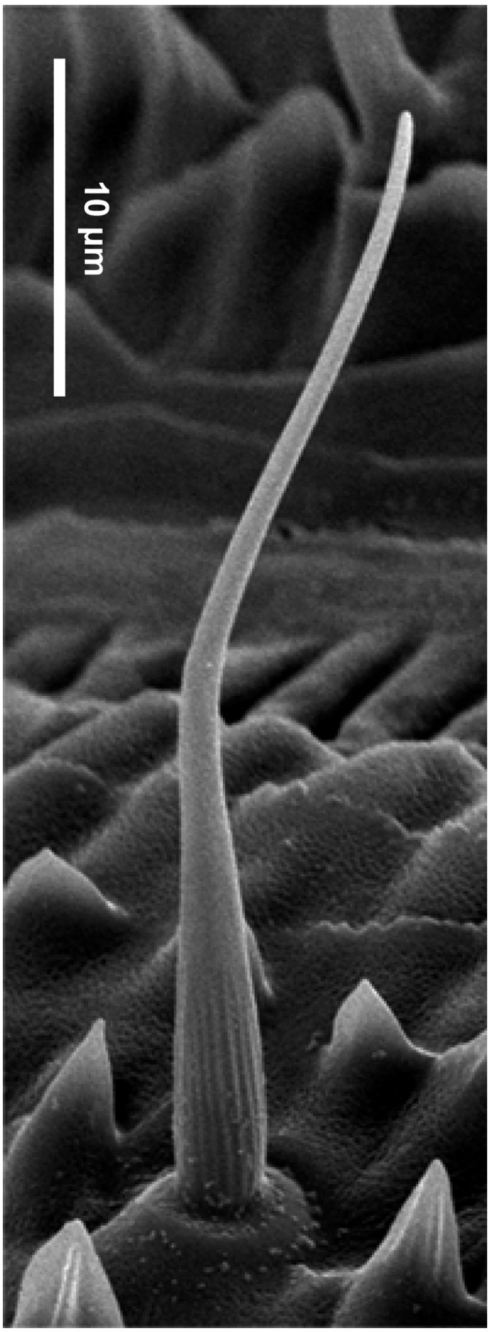

Fig. 1. Single-walled type *C* (*swC*) sensillum associated with an ON and OFF ORN.

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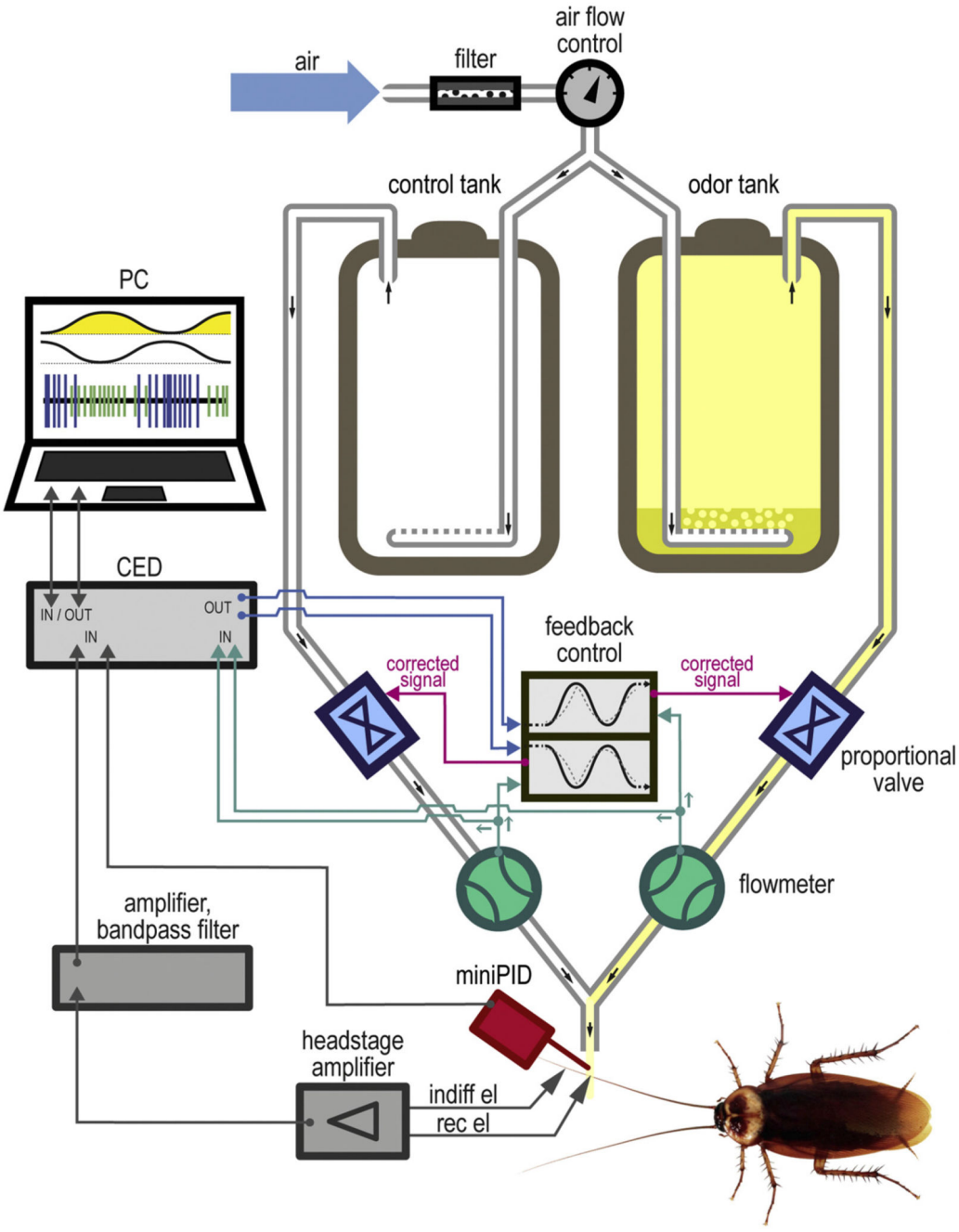

Fig. 2.

Schematic diagram of the dilution flow olfactometer and the electrophysiological recording setup. Odor concentration is varied by changing the flow rate ratios of an odor-loaded and a clean air stream. Mixing ratios are achieved by a pair of oppositely acting proportional valves. Volume flow rates of both air streams are measured by electronic flowmeters. A feedback controller ensures efficient control of the performance of air flow rates. CED (Cambridge Electronic Design) and spike2 software provided data acquisition and

analysis, stimulus sequencing and experimental control. *i* indifferent electrode; *r* recording or different electrode.

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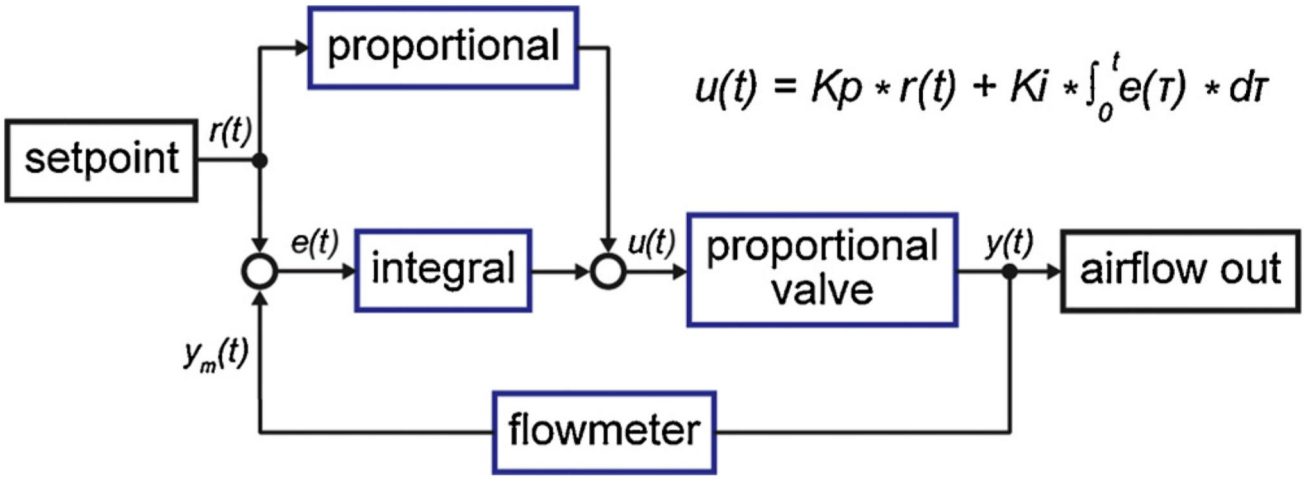

Fig. 3.

PI control block diagram and control algorithm. $r(t)$ reference input, the required setpoint flow rate, $e(t)$ control error as the difference between reference signal $r(t)$ and actual output signal $y(t)$, *Ki* integral gain, *Kp* proportional gain, $u(t)$ controller output signal, sets the valve 's flow rate, $y(t)$ actual output signal, $y_m(t)$ measured flow rate through the valve.

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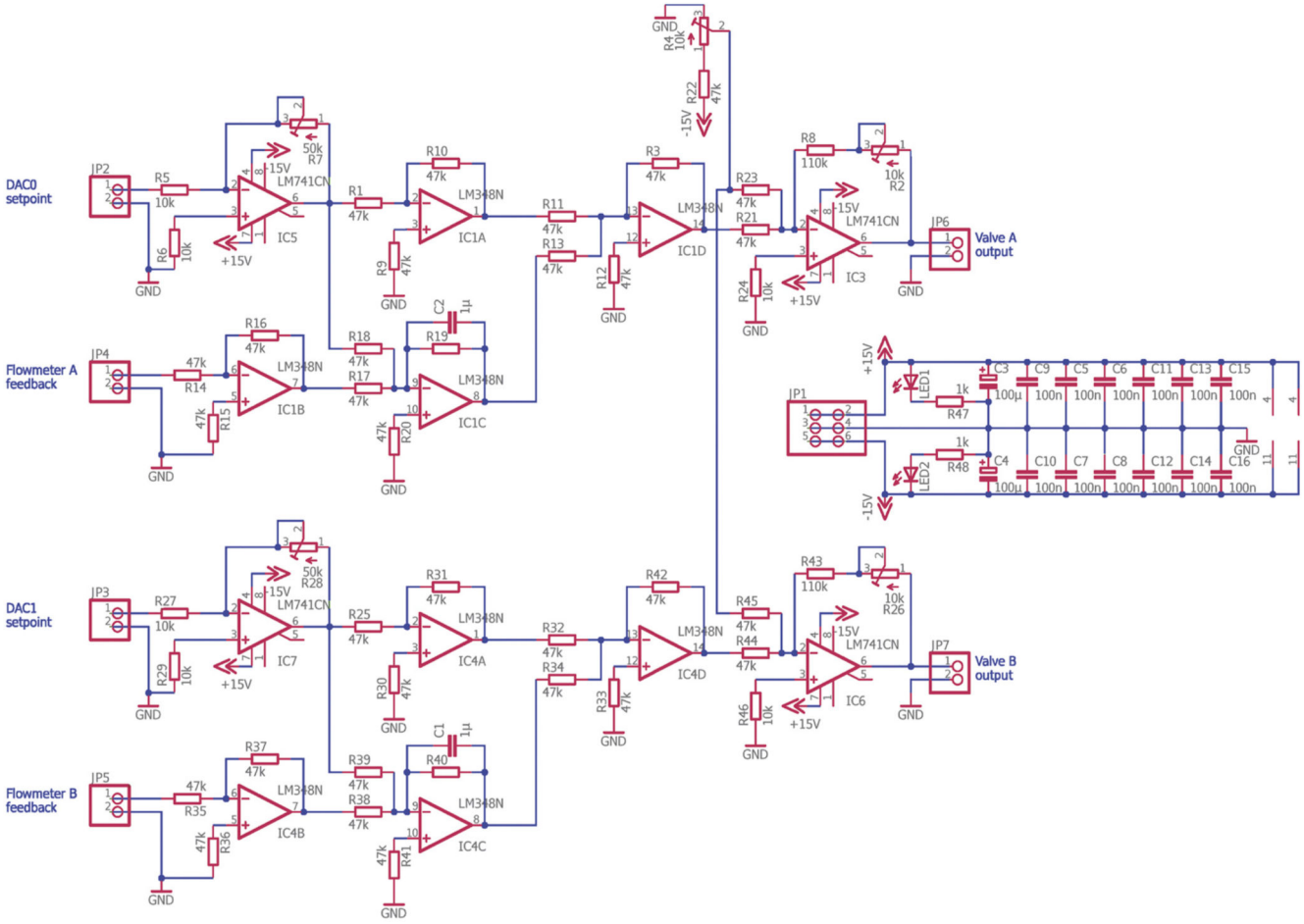

Fig. 4. ***PI*** **controller circuit diagram designed for automatic control of the air flow rate through each proportional valve.**

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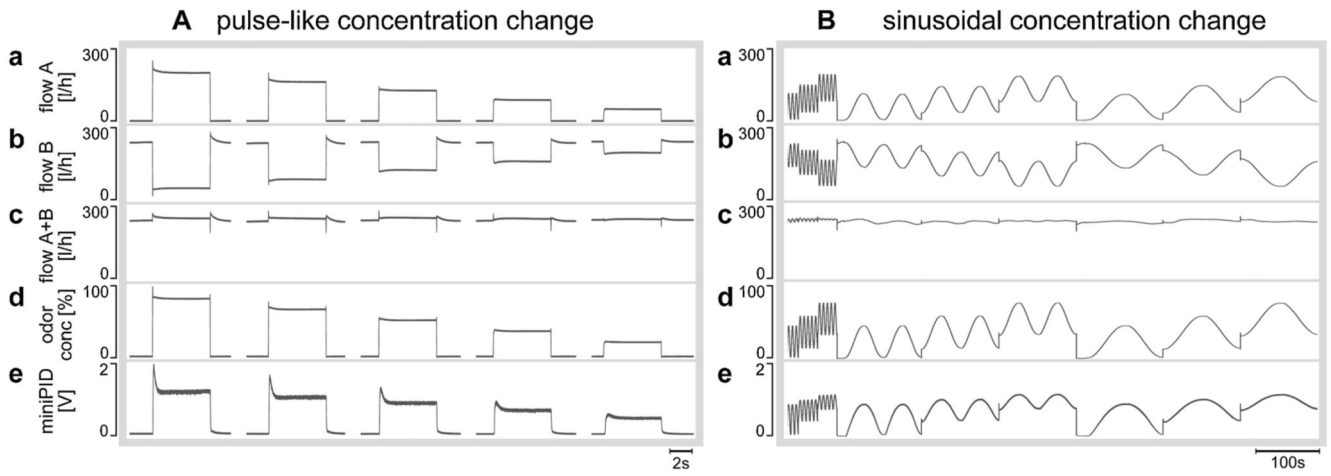

Fig. 5. Continuous output flow of the olfactometer for delivering amplitude-varying concentration pulses and time-varying sinusoidal changes performed at different background concentration levels.

A Series of 5-s concentration pulses with decreasing amplitude. **B** Series of oscillating concentration changes at increasing background concentrations and extending oscillation period. **a** Flowmeter A signals the time course of the odor-saturated air stream controlled by proportional valve A. **b** Flowmeter B signals the time course of the clean air stream controlled by proportional valve B. The control voltages of both valves were 180° phase-shifted. **c** Time course of summed flow rates of the odor-saturated air stream and the clean air stream. **d** Time course of sinusoidal concentration changes determined by the mixing ratio of odor-saturated air in clean air and expressed as percentage of saturated air. **e** Time course of the sinusoidal miniPID signal corresponds with the flowmeter signal in panel a; note slow decrease in peak amplitude with decreasing pulse amplitude.

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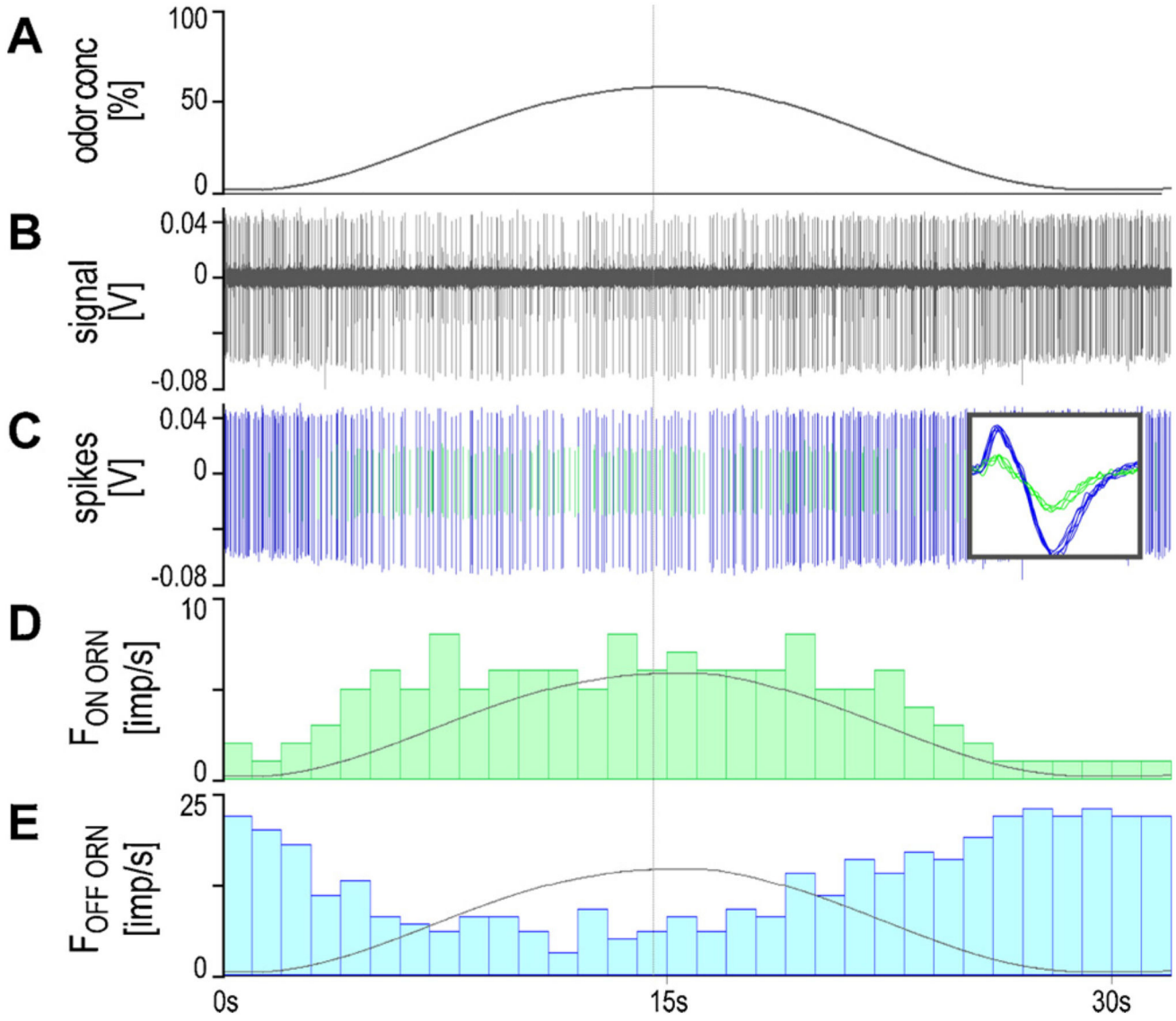

Fig. 6. Antagonistic responses of an ON and OFF ORN to oscillating changes in the lemon oil odor concentration.

A Time course of odor concentration. **B** Simultaneously recorded activity; the OFF ORN generates larger impulse amplitudes than the ON ORN. **C** Off-line sorted action potentials of the ORNs obtained by spike detecting and template matching techniques using Spike2 software (Cambridge Electronic Design, United Kingdom). Green impulses originate from the ON ORN, blue impulses from the OFF ORN. Inset template window shows template boundaries of spike waveforms from the two ORNs. **D, E** Responses of the ON and OFF ORNs represented as time histograms, respectively. Dotted vertical line indicates the phase shift between the maximum concentration of the oscillating odor concentration (**A**) and the maximum oscillating impulse frequency of the ON ORN (**D**) and the minimum oscillating impulse frequency of the OFF ORN (*E*). Plots generated by software spike2.

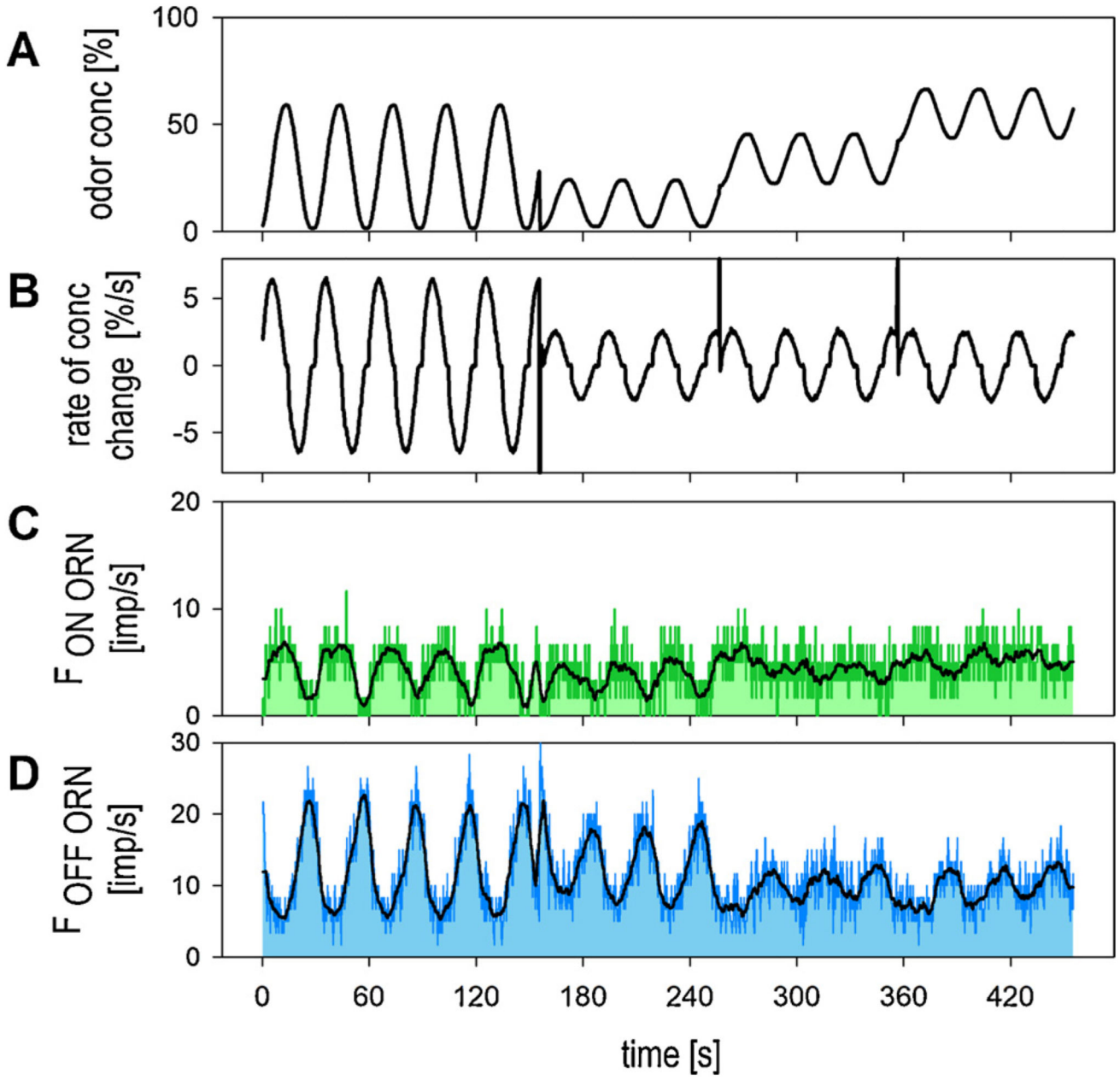

Fig. 7. Antagonistic responses of an ON and OFF ORN to oscillating changes in the concentration of the odor of lemon oil; oscillation period 30 s.

A Time course of odor concentration; large amplitude (60 %) oscillations at zero background concentration are followed by small amplitude (25 %) oscillations at 0% and 25 % background concentration, followed by small amplitude (20 %) oscillations at 45 % background concentration. **B** Time course of the rate of concentration change for the oscillating changes in A. **C, D** Time course of the ON and the OFF cell discharge rates, respectively; bin width 200 ms. The ON-ORN's discharge rate rises with increasing odor concentration; the OFF-ORN's discharge rate rises with decreasing concentration. Plots generated using software spike2.

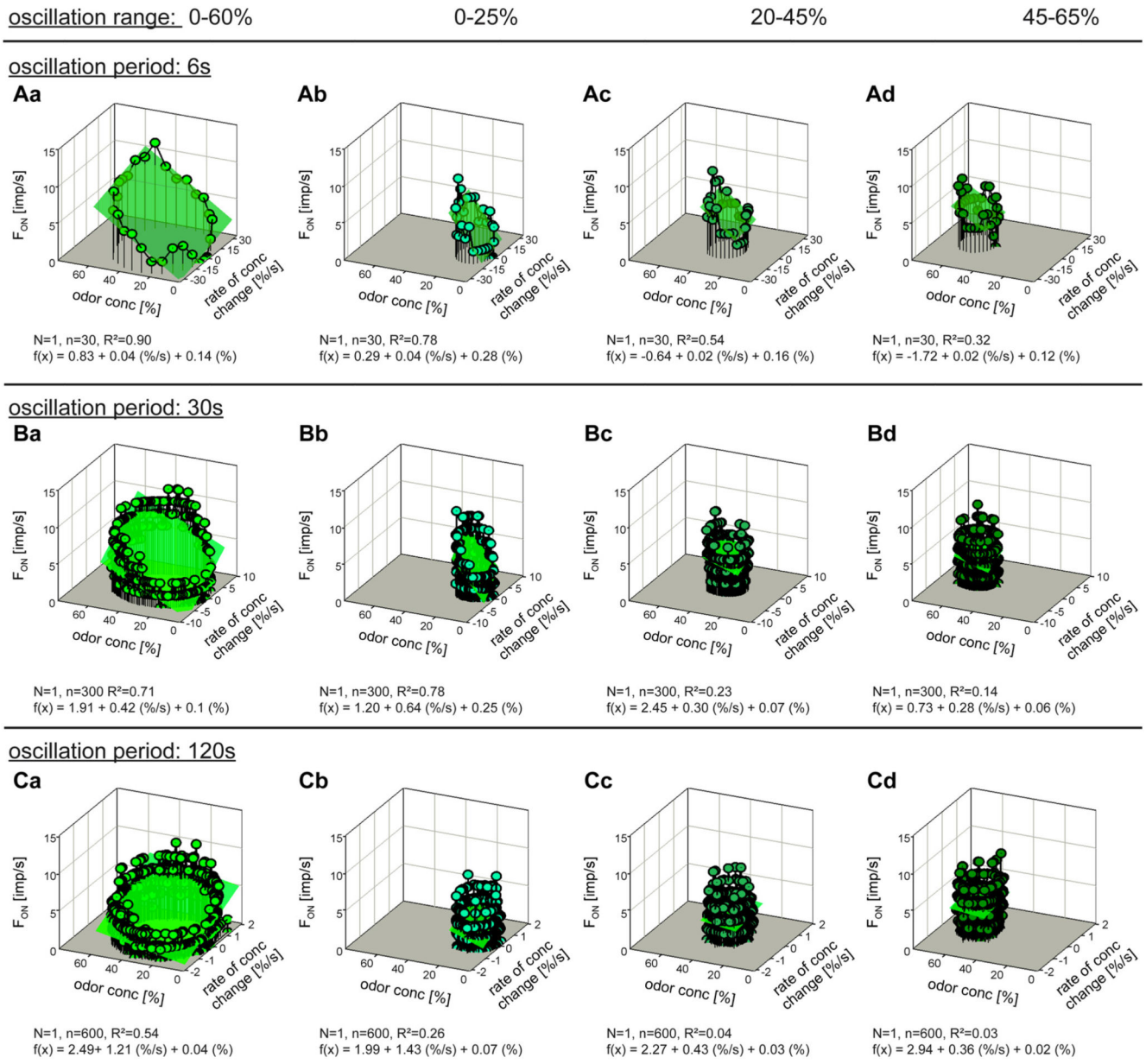

Fig. 8.

Impulse frequencies of a single ON ORN to oscillating concentration changes with two different amplitudes (columns **a, b**) and at three different background concentrations (columns b-d) during three different periods of oscillating concentration changes (rows **A-C**). Impulse frequency plotted as a function of instantaneous odor concentration and its rate of change. Multiple regressions which utilize three-dimensional planes [$F = y_0 + a\,(dC/dt) + bC$; where F is the impulse frequency and y_0 is the height of the regression plane] were calculated to determine the gain of the responses for instantaneous odor concentration (b-slope) and the rate of concentration change (a-slope). Plots generated using SigmaPlot 10.0; bin width 0.2 s; R^2, coefficient of determination.

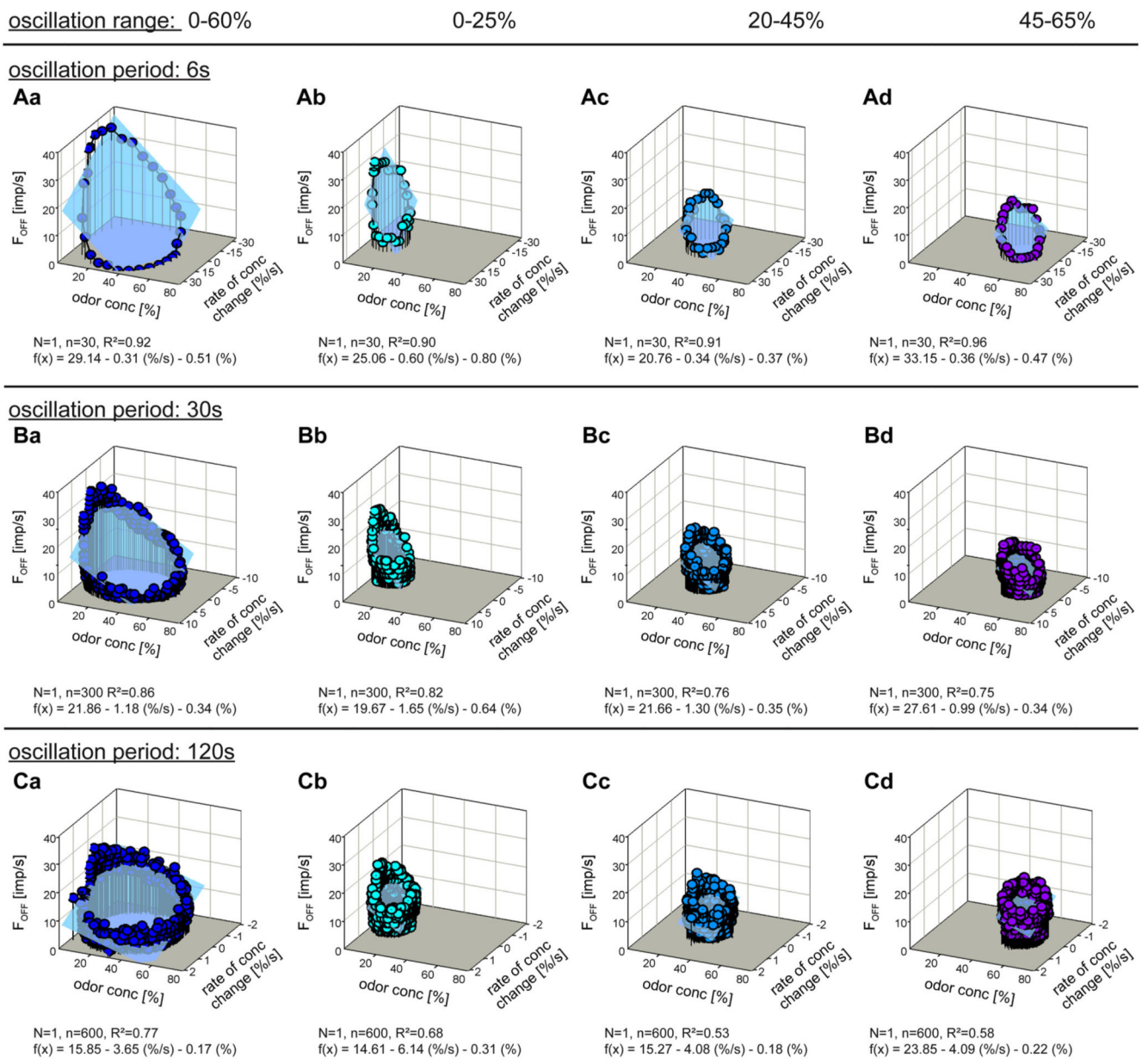

Fig. 9.

Impulse frequencies of a single OFF ORN to oscillating concentration changes with two different amplitudes (columns **a, b**) and at three different background concentrations (columns **b-d**) during three different periods of oscillating concentration changes (rows **A-C**). Impulse frequency plotted as a function of instantaneous odor concentration and its rate of change. Multiple regressions which utilize three-dimensional planes [$F = y_0 + a(dC / dt) + bC$; where F is the impulse frequency and y0 is the height of the regression plane] were calculated to determine the gain of the responses for instantaneous odor concentration (b-slope) and the rate of concentration change (a-slope). Plots generated using SigmaPlot 10.0; bin width 0.2 s; R^2, coefficient of determination.

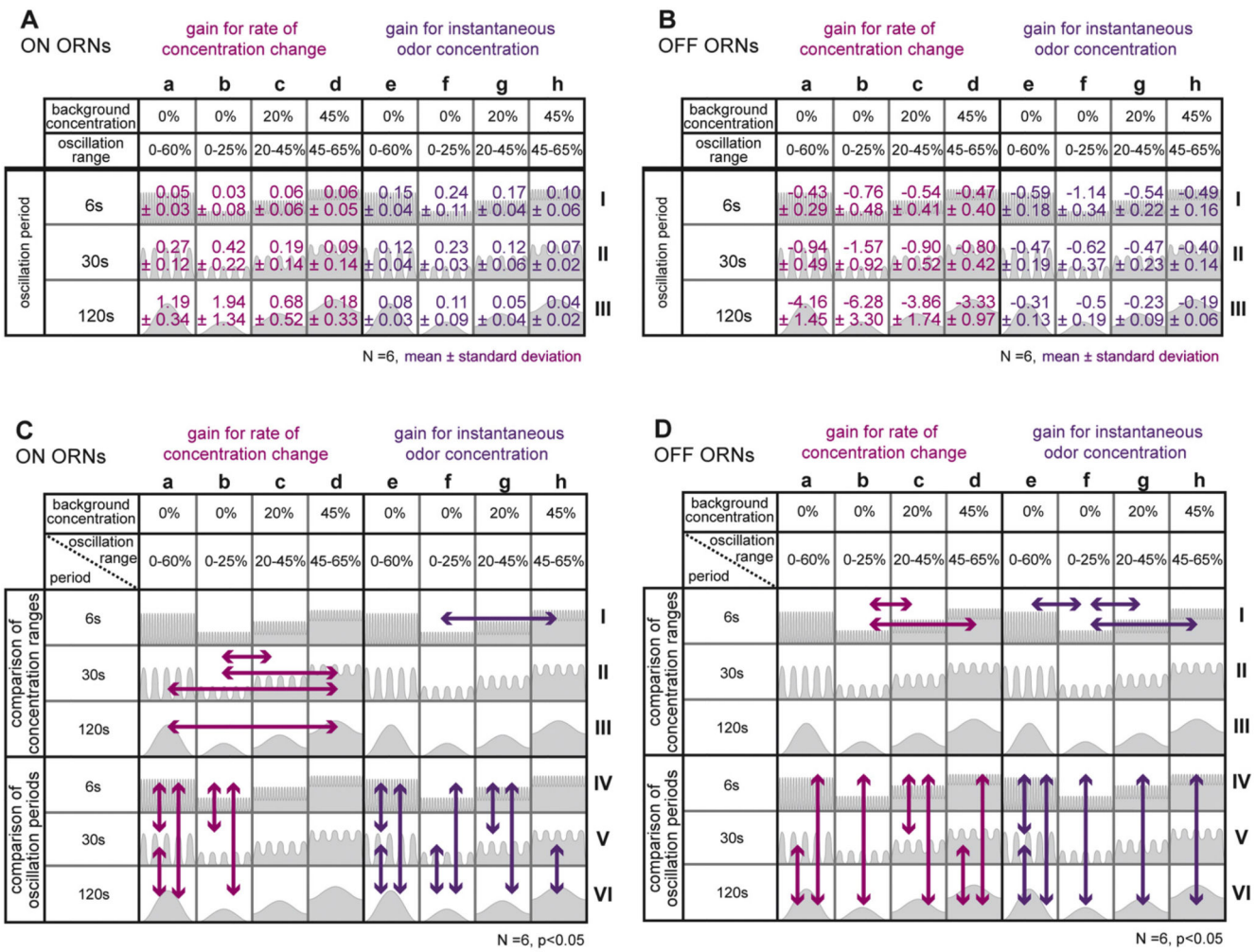

Fig. 10.

A, B Matrix of mean gain and SD for the rate of concentration change and the instantaneous odor concentration pooled from 6 ON ORNs and 6 OFF ORNs for oscillating concentration changes delivered with three different amplitudes at three different background concentrations (*columns*, *a-h*) during three different oscillation periods (*rows*, *I-III*). **C, D** Horizontal double arrows indicate significant differences between two gain values of the rate of concentration change and the instantaneous concentration for oscillation with different amplitudes and at different backgrounds when the oscillation period is constant. Vertical double arrows indicate significant differences between two gain values of the rate of concentration change and the instantaneous concentration for oscillations with constant amplitude and constant background when the oscillation period is varied. Differences between the means were tested by one-way ANOVA, $p < 0.05$.